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The prevalence of antibodies against envelope proteins of Chelonid herpesvirus 5 is inconsistent with the current understanding of the pathogenesis and epidemiology of fibropapillomatosis in marine turtles

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Summary

Fibropapillomatosis (FP) is a neoplastic disease and a most important cause for stranding and death of the green turtle *chelonia mydas*. The Chelonid Herpesvirus 5 (ChHV5) is considered its causative agent. Since ChHV5 does not replicate in cell cultures, many questions concerning its pathogenesis and epidemiology, including the prevalence among marine turtles have not been solved. I developed an ELISA for detecting turtle antibodies against two putative ChHV5 envelope glycoproteins (F-US4 and F-US8). To generate standard antisera for cut-off values, I produced soluble fragments of these antigens with the aim of using them as immunizing antigens in hatchling turtles. To ask from which age on hatchlings would be immunocompetent, I developed a second ELISA, suitable for detecting IgY in turtle sera. It took several months for them to become capable of mounting antibody responses. Therefore, the immunization experiments could not be completed within the time frame of my thesis. I tested a panel of sera collected from Australian and Hawaiian green turtles with or without FP for antibodies against F-US4 and F-US8. I detected only a few animals (almost exclusively with severe FP) with considerable amounts of antibodies against these two antigens and only few animals without FP were identified as carriers of antibodies against ChHV5 antigens. My data do not agree with the present views concerning the causative link between FP and ChHV5 as well as the natural reservoir of ChHV5.

Keywords: Fibropapillomatosis, Chelonid Herpesvirus 5, ELISA, seroprevalence

Zusammenfassung

ZusammenfassungFibropapillomatose (FP) ist eine neoplastische Erkrankung und eine der häufigsten Todesursachen der grünen Meeresschildkröte *chelonia mydas.* Das Schildkröten Herpesvirus 5 (ChHV5) wird als ursächliches Agens angenommen. Da es sich nicht in Zellkulturen repliziert, sind viele Fragen bezüglich Pathogenese und Epidemiologie, so auch die Prävalenz des Virus in der Schildkrötenpopulation noch ungeklärt. Ich habe einen ELISA zum Nachweis von Antikörpern gegen zwei mutmassliche Hüllenglykoproteine (F-US4 und F-US8) entwickelt. Um Standardseren für cut-off Werte zu gewinnen, habe ich lösliche Fragmente der beiden Glykoproteine hergestellt, mit dem Ziel, sie in Immunisierungsexperimenten an jungen Meeresschildkröten einzusetzen. Der Zeitpunkt der Immunokompetenz wurde mit einem zweiten von mir entwickelten ELISA zur Detektion von IgY in Schildkrötenseren bestimmt und betrug mehrere Monate nach dem Schlüpfen, weshalb die Immunisierungsexperimente während meiner Dissertation nicht beendet werden konnten. Ich habe Seren von hawaiianischen, als auch australischen grünen Meeresschildkröten mit und ohne FP auf das Vorhandensein von Antikörpern gegen F-US4 und F-US8 getestet. Fast ausschliesslich stark an FP erkrankte Individuen, enthielten beachtliche Mengen an Antikörpern, und sehr wenige klinisch gesunde Tiere wurden als Antikörperträger identifiziert. Diese Daten stellen die ursächliche Verbindung zwischen ChHV5 und FP sowie dessen natürliches Reservoir in Frage.

Keywords: Fibropapillomatose, Schildkröten Herpesvirus 5, ELISA, Seroprävalenz

Introduction

All seven species of marine turtles, including the Green turtle *Chelonia mydas*, are threatened by different factors including urbanization, environmental pollution, entanglement in fishing nets, hunting and nest and hatchling depredation by wild (van Mil, 2014, Jones et al., 2015). Fibropapillomatosis (FP), a globally distributed, emerging infectious disease, is one of the newer threats reported in every species of marine turtles (Herbst, 1994), but most common in green turtles (Whitehouse, 2015). It is one of the most important causes for stranding and death (Work et al., 2015). FP is a neoplastic disease, characterized by the growth of seemingly benign tumors. The tumors emerge predominantly externally on the skin and on mucous membranes, where they may reduce vision, obstruct feeding and breathing and affect motion (Work et al., 2015; Aguirre et al., 2002). Less frequently, internal tumors are also observed, located in heart, kidney, intestines, and lungs (Work et al., 2015).

The Chelonid Herpesvirus 5 (ChHV5) is believed to be the causative agent of FP but the expression of the disease may require a multifactorial interplay between ChHV5, environmental factors, such as polluted areas (Aguirre and Lutz, 2004), and the host (García-Sastre, Sansonetti, 2010). A relationship between immunosuppression and FP, causing higher susceptibility to secondary infections, has previously been suggested (Work et al., 2001 & 2003). The disease is mostly found in juveniles, less commonly in adults (Page-Korjian et al., 2014, Ene et al., 2005). Prevalence estimates based upon FP records is geographically very variable (Alfaro-Núñez et al., 2014). Prevalence in Hawaii has decreased since 1990 (Work et al., 2015), elsewhere FP has remained stable or even increased (Whitehouse, 2015). However, the way of transmission is still not fully understood. Besides direct contact with infected animals, contact with Herpesvirus-contaminated substrates or through vectors like parasitic leeches or cleaner fishes were suggested (Lu et al., 2000b, Greenblatt, 2004, Whitehouse, 2015). Results of a recent study proposed that transmission of the virus depends on superspreaders, a small number of highly infectious animals (Work et al., 2015).

Chelonid herpesvirus 5 is one of six known herpesviruses in chelonids (1, 5 and 6 in marine turtles; Tidona, Darai, 2011; Jones et al., 2015). According to its genomic structure, featuring a unique long (UL) sequence and a unique short (US) sequence, which is flanked by inverted repeats, (Ackermann et al., 2012), ChHV5 belongs to the genus *Scutavirus* within the *Alphaherpesvirinae* subfamily of the *Herpesviridae* (Davison and McGeoch, 2010).

As yet, it has not been possible to make ChHV5 replicate in conventional cell cultures, although replicating virus has been detected in the epithelial layer of organotypic raft cultures that originated from ChHV5-positive fibroblasts and ChHV5-negative epithelial cells (Work et al., 2017). Therefore, to date not much is known about the virus itself, its pathogenesis, transmission and epidemiology. However, such knowledge is needed in order to understand the disease and to develop curative or preventive strategies, like antiviral treatments and/or vaccines. Development of serological tools would be particularly important to better understand the epidemiology and transmission of the infection and its associated disease.

In the absence of suitable cell culture systems for serial virus propagation, both virus isolation and serum neutralization tests (SNT) cannot be accomplished. Also virus purification for conventional ELISA-antigen production is not feasible for the same reason. A previous attempt to detecting ChHV5-specific antibodies by ELISA used baculovirus-expressed ChHV5 glycoproteine H (gH) as an antigen (Herbst et al., 2008). The results showed that captive turtles

without previous exposure to FP-animals turned seropositive only after cutaneous tumors had appeared as a consequence of inoculation with tumor lysate. These observations suggested that both virus replication and tumor development were necessary preconditions for seroconversion. However, the same study found also consistently up to 80% seropositive animals among free-living turtles in Florida, even if they originated from sites without history of FP (Herbst et al., 2008). Thus, it remained unclear, whether the FP agent ChHV5 itself or a serologically related, non-tumor-causing virus was highly prevalent among healthy turtles in Florida.

As herpesviruses have numerous envelope glycoproteins, each of which having their individual evolutionary pressure, we selected two different viral glycoproteins, i.e. F-US4 and F-US8 (Ackermann et al., 2012), to newly address the question about ChHV5 prevalence among turtles with or without FP. Furthermore, we wanted to allow antigen coating under non-denaturing conditions. Similar to serological assays for papillomavirus antibodies (Sehr et al, 2001), we used Glutathione conjugated to casein as the coating substrate and constructed baculoviruses producing soluble fragments of ChHV5 glycoproteins with C-terminal glutathione-S-transferase (GST) tags. These fusion proteins were expected to bind under native conditions to the previously coated glutathione in order to serve there as ELISA antigens.

As reviewed earlier (Jacobson & Origgi, 2002), sea turtles have at least three classes of immunoglobulins, a 17S IgM, a 7S IgY, and a 5.7 S IgY. The 17S antibodies are considered to indicate a fresh infection, whereas 7S IgY appear some weeks later and 5.7S IgY emerge only several months after the immunogenic event. Ideally, one would look for 17S IgM antibodies in order to detect a fresh infection. Unfortunately, specific antibodies against 17 S IgM are not commercially available. Yet, monoclonal antibodies against 7S IgY and 5.7S IgY were recently developed and made available to us (Work et al., J. Immunol. 2015).

While these antigens and monoclonal antibodies would provide us with essential tools for developing a serological assay, a major problem remained in positive and negative controls. According to Herbst and others (2008), turtles raised in captivity without history of FP might provide suitable negative control sera. However, no truly positive antisera against either F-US4 or F-US8 existed at the time. Therefore, we planned for experimental vaccination of seronegative turtles, using purified derivates of either F-US4 or F-US8. For this purpose another series of baculoviruses was constructed with the aim to produce soluble F-US4 or F-US8 exodomains, respectively, but this time fused to C-terminal c-myc- and 6xhis-tags. The c-myc-tag was selected to identify and characterize the expressed protein by making use of a commercially available monoclonal antibody against c-myc (Young et al., 2012). The 6xhis-tag was to be used for purification of these proteins prior to turtle immunization (Dodson et al., 2007; Young et al., 2012).

Our permit issued by Australian authorities for vaccinating turtles with our experimental vaccines was restricted to the use of turtle hatchlings. However, to date not much is known about the development of the immune system in turtles and it was not known at which age, the newly born turtles would reach the immunological competence to produce their own antibodies upon vaccination. To address this question, we developed an ELISA to test hatchling's sera for the presence and quantity of 7S and 5.7S IgY in their serum.

Using these approaches, we found that it takes turtles several months following hatching to reach a degree of immunological maturity that makes it worthwhile to vaccinate them. The antigens intended for use in experimental vaccinations could be purified up to a degree of

approximately 80%. Our serological survey using the newly developed ELISAs for detecting antibodies against F-US4 and F-US8, respectively, confirmed that captive-reared turtles without FP remained seronegative. Yet, in contrast to Herbst (2008), we found that free ranging turtles from Hawaii and from Australia exhibited very rarely a strong antibody response against either viral protein.

Aims of the present work

The first aim of the present work was to develop an ELISA for detecting turtle antibodies against two selected ChHV5 antigens, namely F-US4 and/or F-US8. The assay would then be validated by using sera from captive turtles without history of FP as negative controls and sera from turtles with FP as potential positive controls. Eventually, consecutive sera from F-US4- and/or F-US8-vaccinated turtles would serve as means to establish the cut-off between seropositive and seronegative.
To get new insights into the epidemiology of ChHV5, I wanted to analyze a panel of turtle sera from the wild, including samples from areas with reportedly high frequency of FP and from areas without history of FP cases. Moreover, I wanted to test sera from animals with various degrees of FP.

Furthermore, I wanted to establish an ELISA for quantifying 7S and 5.7S IgY in the sera of hatchlings and juvenile turtles, respectively, and to find the proper developmental stage for starting the immunization experiment. A panel of hatchling sera and a panel of juvenile turtle sera from various locations in Australia should be used for that purpose.

Materials and Methods

Cloning Strategy

In order to express differently tagged glycoprotein fragments from a recombinant baculovirus vector, the Gateway donor vector pDONR221 was modified to provide an N-terminal baculovirus signal sequence and a C-terminal c-myc-GST-tag to our proteins of interest. This vector, designated pDONR221-Signal-Bam-Cmyc-GST, was opened by BamHI digestions to accommodate our constructs of interest, which were also synthetic and came with BamHI sites at both of their designated ends. Once the correct sequence and orientation of the inserted fragments had been verified, each construct was transferred by LR recombination to the Gateway expression vector pDEST8, which added flanking sequences to the construct that were needed for its recombination into the polyhedrin locus of BACMID-cloned baculovirus sequences. Finally, baculoviruses were reconstituted by transfecting the BACMID DNAs to Sf9 cells.

Construction of pDONR221-Signal-Bam-Cmyc-GST

For this purpose, a synthetic sequence was ordered from GenScript (http://www.genscript.com), comprising an attB1 sequence, followed by the SP1–2 signal peptide sequence (Futatsumori-Sugai and Tsumoto, 2010), a BamHI site, a c-myc-tag, a codon-optimized GST-sequence, and an attB2 sequence. The fragment was amplified by PCR (PHusion DNA Polymerase from Thermo Scientific, Primers were ordered at Microsynth) and subsequently gel purified (For Agarose gel electrophoresis, Gene Ruler Mix DNA ladder from Thermo Scientific as well as the 1kb DNA ladder from Biolabs were used). In a total volume of 8 µl, 150 ng of the PCR product were mixed with 180 ng of purified pDONR221 DNA (Life technologies) and 4.5 µl TE buffer pH 8.0. Then, 2 µl BP clonase II enzyme mix (life technologies) was added and the recombination step was carried out according to the standard Gateway protocols. The BP recombination reaction facilitates transfer of a gene of interest-in this case the attB_Signal_C-myc_GST PCR product-to an attP containing vector as pDONR221. Finally, DH5α Chemically Competent E. coli were transformed with the BP reaction mix and recombinant progeny was selected on LB containing 50 µg/ml kanamycin. To enable selection of entry clones, the pDONR221 vector on the one hand contains a Kanamycin resistance gene, on the other hand the two att sites are flanking a cassette containing the ccdB gene for negative selection.

Construction of the entry clones

Purified **pDONR221-Signal-Bam-Cmyc-GST** DNA was BamHI (Roche) digested and phosphatase-treated (Biolabs). Also the synthetic vectors containing the genes of interest (ordered at GenScript) were digested with BamHI. In order to produce the entry clones, T4 ligations (T4 ligase was purchased from Biolabs) of the BamHI digested vector **pDONR221-Signal-Bam-Cmyc-GST** with the inserts flanked by BamHI restriction sites were carried out. Afterwards, DH5α Chemically Competent E. coli were transformed with the ligation reactions and entry clones were selected on LB containing 50 µg/ml kanamycin.

Construction of the expression clones

DNA of the selected entry clones was extracted and purified. In a volume of 8 µl, 150 ng of the entry clone DNA were mixed with 200 ng of purified pDEST8 vector DNA (Life technologies) and 5.2 µl TE buffer pH 8.0. Then, 2 µl LR clonase enzyme mix (Life technologies) was added and the recombination step was carried out according to the Gateway protocols. The LR recombination reaction facilitates transfer of a gene of interest-in this case the attL_Signal_C-myc_GST cassette containing the synthetic glycoprotein-gene insert - to an attR containing Destination vector as pDEST8. Subsequently, DH5α Chemically Competent E. coli were transformed with the LR reaction mix and recombinant progeny was selected on LB containing 100 µg/ml ampicillin. To enable selection of expression clones after Transformation, the pDEST8 vector has similar selection properties as the pDONR221 vector used for the BP reaction, namely the Ampicillin resistance gene and a ccdB gene flanked by the att sites, respectively.

Production of the bacmid DNA

The pure expression clones were used to transform MAX Efficiency® DH10Bac™ competent cells (Life technologies) for transposition of the genes of interest into the bacmid (Life technologies). To identify the colonies containing the recombinant bacmid, an antibiotic selection and blue/white screening was used. Kanamycin resistance is conferred by the parent bacmid (bMON14272), the helper plasmid (pMON7124) contains the tetracycline resistance gene, and the gentamycin resistance gene is carried by the pDEST8 vector.

Reconstitution of recombinant baculoviruses

Cell Lines:

The Sf9 insect cells (Life technologies), used for viral stock production and Endpoint-dilution experiments, were cultured in TNM-FH (Grace's Insect cell medium supplemented (Life Technologies) containing 10% FCS). Mimic Sf9 Insect cells (Life technologies) were used for protein expression and they were cultured in serum-free medium Sf-900 III SFM (Life Technologies). Both cell lines were cultured at 27°C.

Baculoviruses were reconstituted by transfecting the bacmid DNAs to Sf9 cells. In order to generate infectious baculoviruses, purified bacmid DNAs were transfected to Sf9 cells. 1µg of DNA and 4µl of the FuGene transfection reagent (Promega) were added to 25 µl of Grace's Insect Medium unsupplemented (life technologies). The whole mix was transferred to a tube containing 500 µl of TNM-FH medium (Grace's Insect cell medium supplemented (Life Technologies) containing 10% FCS) and then the mixture was added to 200'000 Sf9 cells attached to one well of a 24-well plate. Cells were incubated at 27°C. After 96 hours medium was collected, centrifuged at 1000g, 4°C for 10 minutes. Supernatant was harvested as p0 viral stock solution. An Aliquot of 200 µl was frozen down at -80°C for storage, the rest was kept at 4°C for further propagation of the viral stocks. Transfected cells were detected by means of Immunostaining at different time points post transfection, namely 72 h, 96 h and 120 h. Cells were fixed in 4% PFA for 15 minutes, blocked and permeabilised in PBS, 0.1% Triton-X100, 3% Horse serum and 1% BSA. Cells were then stained using monoclonal mouse anti V5 primary antibody (1:500, overnight at 4°C (Invitrogen)) or monoclonal mouse anti C-myc primary antibody respectively (1:500, overnight at 4°C (Invitrogen)) and alexa fluor 488 goat-anti-mouse monoclonal secondary antibody (Life technologies). Nuclei were stained using

DAPI (life technologies, 1:1000, incubated for 15 minutes) Green fluorescent cells represent infected cells expressing the recombinant protein containing the V5 tag or the C-myc tag respectively.

With the aim of baculoviral p1 amplification, Sf9 cells were infected with the p0 viral stock solution and incubated at 27°C. For that purpose, 4 x 10^6 Sf9 cells cultured in 5 ml TNM-FH medium were infected with 100 µl of the p0 viral stock solution and incubated at 27°C. The p1 viral stock was harvested 7 days after infection. P2 baculoviral amplification was carried out the same way using the p1 viral stock solution and Sf9 cells.

To estimate the viral titer of the p2 viral stock an end-point dilution experiment was carried out. For that purpose, Sf9 cells were seeded on a 96-well plate (40'000 cells per well) and infected with 100 µl of TNM-FH containing different dilutions of the p2 viral stock (1- 10^{-15}) and incubated at 27°C for 96 hours before fixing the cells and staining them using the Immune Peroxidase technique with the primary antibody anti-V5 mouse mAb (1:1000, 45 minutes at 37°C) (or anti-C-myc antibody mAb (1:1000, 45 minutes at 37°C)) and the secondary HRP-linked goat anti mouse IgG (H+L)-HRP (1:1000, 45 minutes at 37°C(thermofisher). As a substrate, AEC substrate was used (6% AEC-solution (20mg AEC in 3 ml dimethylformamide) in 0.05 M Acetate buffer containing 0.1% H_2O_2). Red stained cells represented infected cells expressing the recombinant protein containing either the V5 tag or the C-myc tag.

Protein expression and purification

In order to verify expression of the desired proteins, immunostaining of the fixed cells using monoclonal anti-V5 antibody and monoclonal anti-C-myc antibody, respectively, revealed presence of the desired protein in Baculovirus-infected cells. Additionally, the secreted form of the protein was detected by Western immunoblotting of cell supernatant by staining the PVDF membrane with the monoclonal mouse anti-V5 or the monoclonal mouse anti C-myc primary antibody (Page Ruler Prestained Protein ladder 10-250K from Thermo Scientific was used for PAGE). For detection, the biotinylated horse anti mouse secondary antibody (vector laboratories) and the detection system with the Vectastain ABC Kit (Vectastain) and the substrate 3, 3' – Diaminobenzidine tetrahydrochloride (Sigma) were used. Finally, the secreted V5-5his-tagged proteins produced for immunizing turtles to generate reference sera were pulled down from the supernatant following incubation with magnetic nickel beads (Cube Biotech), elution, and Western immunoblotting with anti-V5 antibody. The C-myc-GST-tagged variants used as ELISA antigens were not purified.

Protein production

For large-scale antigen production, mimic Sf9 cells (obtained from Life technologies) were infected with the P2 viral stock solution at an MOI of 5 (4 x 10^7 cells per T150 flask, 40 ml SFM) and incubated at 27°C for 7 days. Protease inhibitor (Complete mini protease inhibitor from Roche) was added to the medium after harvesting.

To further investigate for protein expression and secretion, medium (P3 viral stock) was analyzed by Western immunoblotting using the anti-V5 antibody (1:5000) and anti-C-myc (1:5000) respectively.

Protein Purification

The secreted protein of the constructs used for immunization were equipped with a C-terminal 5his tag.

Cell medium containing the His-tagged proteins were purified using PureCube His Affinity MagBeads (Cube Biotech).

Medium was added 1 in 7 to binding buffer (50 mM NaH2PO4, 300 mM NaCl, 5mM Imidazole, 10mM β-Mercaptoethanol, pH 8.0) and incubated with the 25 % magnetic bead suspension (1ml/50ml diluted medium, equilibrated in 10 ml binding buffer) at 8°C for 1 hour on an end-over-end shaker. The tube was then placed on a magnetic stand allowing the beads to separate. The supernatant was removed (designated as flow-through fraction). Next, the beads were washed twice with washing buffer (10 ml/ 250 µl beads, 50 mM NaH2PO4, 300 mM NaCl, 10 mM Imidazole, 10mM β-Mercaptoethanol, pH 8.0). The His-tagged protein was eluted using 1 ml of elution buffer/ 250 µl beads (50 mM NaH2PO4, 300 mM NaCl, 60 mM Imidazole, pH 8.0). The fractions were analyzed by SDS-PAGE.

ELISA:

The GST-tagged proteins were used for coating of the ELISA plates. Binding properties were tested using the mouse anti C-myc monoclonal antibody. Different antigens were coated and sera of animals other than turtles were tested in order to establish the appropriate ELISA conditions. A selection of turtle sera were then tested.

ELISA protocol: screening turtle's sera for anti-F-US4/F-US8 7S and 5.7S IgY

Plates for ELISA experiments were NUNC IMMUNO PLATE MAXISORP 439454 from Thermo Scientific.

Wells were coated over night at 4°C with 50 µl linked casein-glutathione (ELISA diluted 1 in 500 in coating buffer (10 mM Na_2CO_3, 40 mM $NaHCO_3$ (casein-glutathione synthesized according to protocol of Sehr, P. and Waterboer, T., January 2006; appropriate dilution determined in a GST-capture ELISA)). Then the wells were washed 3x with 200 µl PBST (0.3 % Tween-20 in PBS). For blocking, 100 µl blocking solution (2 % milkpowder in PBST) was added per well and incubated at 37°C for one hour. Next, the GST-tagged antigen was added (cell medium supernatant containing non-purified protein, diluted in blocking buffer (1:200 for construct F-US4 exofuse C-myc GST, 1:100 for antigen F-US8 exofuse C-myc GST respectively)). The plate containing the antigen was incubated at 37°C for another hour and then washed twice with 200 µl PBST per well and once for 10 minutes with High salt milk (2 % milkpowder in PBS, 0.5 M NaCl). Sera were diluted 1 in 25 in High salt milk and distributed to the wells, 50 µl each. It was again incubated at 37 °C for one hour, followed by three washing steps, 1x with high salt milk and twice with PBST. Monoclonal mouse antibodies (7S CO2; 309 µg/ml (1:1000 in blocking solution); 5.7S D70; 461 µg/ml (1:1500 in blocking solution)) were added and incubated for one hour at 37 °C, 50 µl per well to detect the reaction. After washing (3x with 200 µl PBST), secondary antibody (goat anti mouse HRP conjugated, 1.1000 in blocking buffer (GE Healthcare)) was added, 50 µl/well and incubated 30 minutes at 37°C. Plate was then washed 5x with 200 µl PBST and 1x with PBS to get rid of Tween-20 residues. TMB substrate (Thermo Scientific) was prepared (1:1 in dark) and added to the wells, 100 µl/

well. Reaction was measured after 10 minutes at 650 nm wavelength. Reaction was stopped after 15 minutes using 2M H_2SO_4, 100 µl/well and measured again at 450 nm.

GST-antigen titration:

Same ELISA-protocol, but in this experiment, antigens were added in serial dilutions from 1 in 25 up to 1 in 51200. Instead of sera, anti c-myc mc Ab was added (1 in 5000 in blocking buffer).

Age dependent antibodies in turtle sera:

Same protocol with the following modifications: sera were coated directly to the ELISA plate (coated to dryness over night at room temperature). Protein G (Thermo scientific) was added in place of primary antibody (1:1000 in blocking solution), as secondary antibody, HRP-conjugated mouse anti protein G antibody (Abcam) (1:1000 in blocking buffer) was used.

Sera:

Turtle sera subjected to the ELISA for screening were collected in Australia (Ollera, Toolakea, JCU) (provided by Associate Professor Ellen Ariel (James Cook University, Australia)) and in Hawaii (Big Island, Kaneohe Bay).

Results

Part 1. Construction of recombinant Baculovirus BACMIDs

Synthetic constructs

The Gateway technology and the BAC-to-BAC system were modified to ultimately construct four different baculoviruses for expressing two types of ChHV5 glycoprotein fragments. As outlined in Fig. 1, the predicted exodomains of two different ChHV5 glycoproteins, F-US4 and F-US8, respectively (Fig. 1A), were selected. In a first step (Fig. 1B), the amino acid sequences of these exodomains were reverse translated to a nucleotide sequence, which was optimized for expression in insect cells. At this stage, two variants of each exodomain were synthesized, the first with a C-terminal V5-6his tag, followed by a STOP codon, the second construct without tags and without STOP codons at their 3' ends. All four of these constructs were flanked on both sides with BamHI restriction enzyme sites. In a second step, a synthetic and codon-optimized master cassette was constructed to accommodate each of these four constructs. As outlined in Fig. 1C, this master cassette consisted of att recombination sites on either end, flanking in a 5' to 3' order a synthetic baculovirus signal sequence, a single BamHI restriction enzyme site, a c-myc tag, and a GST-tag. All five constructs were cloned into pUC57 before their nucleotide sequences were verified. The properties, tags, and sizes of the individual constructs are provided in the appendix.

Assembly of Master cassette with exodomains

The master cassette was PCR amplified using primers targeting some 20 nucleotides beyond the att sites (Tab.). The PCR product was purified by agarose gel electrophoresis, excised, and transferred to the Gateway entry vector pDONR221 by the BP reaction. The sequence of the pDONR221 comprising the Master cassette was again verified by sequencing. In a next step, pDONR221 containing the Master cassette was opened by BamHI restriction enzyme digestion and treated with phosphatase. Meanwhile, each of the four other synthetic constructs were excised from pUC57 by BamHI digestion. The resulting fragments were separately gel purified, excised, and ligated together with the linearized and phosphatase treated pDONR221 containing the Master cassette. Four clones, each containing one of the desired glycoprotein exodomains in the desired orientation within the Master cassette, were verified by sequencing prior to further use. These sequences are listed in the appendix.

Transfer to pDEST8

pDEST8 is a Gateway vector suitable to transfer by transposition the desired constructs into the polyhedrin locus of a baculovirus BACMID. The LR recombination reaction was used to transfer each of the pDONR entry clones into the destination vector pDEST8. Once more, all of the successful clones were subjected to sequencing. In three cases, F-US4-C-myc-GST, F-US4-V5-6his, and F-US8-V5-6his, the full sequence could be verified. The fourth construct did not achieve a full sequencing overlap, leaving a gap of estimated 360 bp, which could not be verified. But the correct orientation of insertion could be proved. All four constructs were used for transposition.

Transposition into Baculovirus BACMIDs

Maxiprep DNA from each construct was used to transform competent DH10Bac cells for transposition of the genes of interest into the baculovirus BACMID.

Two white colonies of each construct were picked and restreaked on a fresh plate. Since all new colonies were white, one colony of each construct was chosen for amplification and further characterization.

A PCR using the primers pUCM13 forward and pUCM13 reverse (Tab.) was carried out and the PCR products were separated by agarose gel electrophoresis. In the absence of an insert, a product of approximately 300 bp was expected. In the present case, however, PCR products ranging from approximately 3800 to 4600 were observed, which essentially matched the expectations for Sig-F-US4-V5-6his (3980 bp), Sig-F-US4-c-myc-GST (3850 bp), Sig-F-US8-V5-6his (4650 bp), and Sig-F-US8-c-myc-GST (4530 bp), respectively. Accordingly, DNA was prepared for transfection experiments.

Reconstitution of recombinant Baculoviruses

Proteins of interest in fixed insect cells

With the aim of generating infectious baculovirus from the BACMID-DNA of the four constructs, conventional sf9 cells were transfected using 1 µg DNA for the his –tagged constructs and 2 µg DNA for the GST-tagged ones, respectively. Due to the knock-out of the polyhedron gene, plaques indicative for recombinant baculovirus replication were difficult to detect. Therefore, the transfected cells were investigated using immunofluorescence using antibodies against the protein tags. At 96 hrs post transfection, the cell supernatant was harvested as the p0 viral stock. The cells were fixed and incubated with monoclonal antibodies (mAb against V5 or mAb against C-myc) before addition of a goat-anti-mouse IgG, conjugated with alexa fluor 488, to provide a green fluorescence, while the nuclei were counterstained with DAPI. An exemplary result is shown in Fig. 2. Approximately 5% of the cells transfected with Signal-F-US4-V5-6his provided a bright green fluorescence upon staining with the mcAb against V5, indicating that the protein of interest was being synthesized in the successfully transfected cells. Similar results were achieved with the other three constructs (data not shown), indicating that a four desired baculoviruses had been generated. The same immunostaining strategy was used for limiting dilution assays of the baculovirus stocks in order to determine the viral titers. Tab. shows the titers achieved for P0, P1 and P2 of each construct.

Part 2. Purification of baculovirus-expressed proteins for immunization

Proteins of interest in infected cell supernatants

According to our strategy, the proteins to be used for immunization of turtles, had been truncated at their C-terminus, prior to their transmembrane domains and had been supplemented with a synthetic baculovirus signal sequence, which was expected to guide the new proteins to the secretory pathway. Accordingly, a considerable portion of these proteins was supposed to be secreted to the cell culture supernatant, from which it could be purified. To test this assumption, conventional Sf9 cells or, alternatively, mimic Sf9 cells were inoculated with P2

of the baculoviruses at MOI5 and cell-free supernatants were harvested at 5 days post infection. A sample of each supernatant was separated on a polyacrylamide gel and transferred to a PVDF membrane, before being stained with anti-V5 mcAb as described in materials and methods.

As shown in Fig. 3, F-US4-V5-6his provided double bands, migrating in between of the 25 kDa and 35 kDa markers. The observed mobility corresponded well with the predicted molecular weight of 24.5 kDa. While the two lower bands migrated in a similar manner, the upper band migrated slightly slower, when the virus was grown in mimic Sf9 cells as compared to the virus grown in conventional Sf9 cells. These observations suggested that the faster bands represented the non-glycosylated precursor, whereas the slower band represented glycosylated forms of the secreted fusion proteins. The additional size difference between the two slower bands may be attributed to the differential glycosylation strategies of the conventional Sf9 insect cells versus the humanized mimic Sf9 cells. Moreover, these results confirmed that the desired F-US4 fusion protein was actually secreted to the supernatant of the infected cells.

Similar results were obtained for the F-US8-V5-6his fusion protein (Fig. 3, lanes 3 and 4). A broad band migrated in between of the 55 and the 70 kDa marker bands, which fits well with the predicted molecular weight of 50.1 kDa F-US8-fusion protein. While in this instance the unglycosylated precursor did not clearly separate from the glycosylated forms of the protein, the fuzzy quality of the band was in accordance with the expected behavior of a glycoprotein. The fuzzy quality of these bands did not allow to discriminate differential glycosylation due to conventional or mimic Sf9 cells, respectively. In addition to the expected mature form of the protein, several bands running at around 15 kDa may represent break-down products of the fusion protein. However, also the F-US8 fusion protein was secreted to the cell-free supernatant.

Ni-purification of F-US4- and F-US8- fusionproteins

Apart from the V5-tag, the F-US4- and F-US8 fusion proteins were also equipped with a C-terminal 6his-tag, which had been included to facilitate protein purification.

After optimizing the conditions for purifying the desired proteins on magnetic nickel beads (data not shown), T150 flasks of Sf9 or mimic Sf9, respectively, cultures were inoculated at MOI5 and cell-free supernatants were harvested at dpi 7. After conditioning the supernatants as described in Materials and Methods, they were incubated with nickel beads, washed, and eluted with imidazole. Samples of flow-through, final wash, and eluate were separated by PAGE and transferred to PVDF membrane for immunoblotting (Fig. 4). Alternatively, samples of the starting materials and eluates were subjected to silver staining (Fig. 5). As the exemplary results in Fig. 4 and 5 indicate, the desired fusion proteins were quantitatively removed from the starting materials and not present in the final wash but constituted >90% of the protein in the elution fraction.

Using this approach a total of approximately 30 mg purified F-US4 and 40 mg purified F-US8 protein were prepared.

Part 3. Analysis of the turtle's seroresponse against ChHV5

Age-dependence of antibodies in turtle sera

While monoclonal antibodies against various turtle IgY exist (Work et al., J. Immunol. 2015), little is known about the presence of these IgY at different development stages of the turtles. Since we wanted to analyze sera of hatchlings for antibodies against ChHV5, it was of interest to know, which types of antibodies were actually present in the sera of hatchlings at the age of a few weeks. To address this question, we coated the sera of hatchlings (n=12) as well as of juvenile (n=22) turtles to ELISA plates. Since monoclonal antibodies against 7S (mcAb CO2) as well as 5.7S IgY (mcAb D70) were available to us and since it is known that protein G is able to bind some turtle IgY (Work et al., 2016), we coated each serum in triplicate and also in two different dilutions, i.e. 1:100 and 1:1000. Human serum and milk, respectively were used as positive and negative controls, particularly for protein G but also for establishing the specificity of the anti-turtle IgY antibodies in this test. The ELISA was carried out as described in Materials and Methods and results are shown in Fig. 6. Using the CO2 mcAb (Fig. 6A), a median optical density (OD) of 1 was detected with the hatchling sera coated at 1:100, a value, which was reduced to OD 0.35 upon coating at 1:1000. Upon coating of the sera at 1:100 from juveniles a median OD of 2.6 was achieved, a value, which was not significantly reduced upon the 1:1000 coating. Thus, the ELISA was able to detect 7S IgY in the coated sera of both hatchlings and juveniles. However, these observations also suggest that the maximum coating capacity of the ELISA plate had been achieved with both dilutions when sera from juveniles were used. In contrast, the hatchling's sera did not contain enough 7S IgY to coat the ELISA well to saturation. The OD value was significantly reduced at the 1:1000 dilution. Upon using the D70 mcAb against 5.7S IgY (Fig. 6B), a median OD of 3.1 was observed with the sera from juveniles, a value, which was significantly reduced to OD 2.8 upon the 1:1000 dilution. In contrast, the sera from hatchlings achieved median ODs of 0.18 (1:100) and 0.078 (1:1000), which were not significantly different. These observations suggest that the ELISA did not or hardly detect 5.7S IgY in the sera of hatchlings, whereas 5.7S IgY were abundantly present in the sera of the juveniles. As expected, protein G (Fig. 6C) was able to detect some immunoglobulin in the turtle sera, although only in the ones from juveniles (median OD 0.59) and only if they were coated at 1:100. Hatchling sera at both dilutions as well as juvenile sera at 1:1000 provided only background values in the ELISA (median values at 0.08 to 0.09). While human sera were not recognized by either the CO2 or the D70 mcAb, protein G achieved an OD value of approximately 1.5 at a dilution of 1:100 and of 1.0 at 1:1000 (data not included in the Figure). In contrast, only background levels were achieved with all visualization systems, when milk was coated (data not included in the Figure). The serum of one single, several months old turtle had also been analyzed in this ELISA but the results were not included in the Figure because, due to its age, it did not match into any of the two groups. Interestingly, the results presented actually an intermediate between the two groups, suggesting that both 7S and 5.7S IgY develop rapidly during the first few months of life.

In summary, 7S IgY were detected in the sera of both hatchlings and juveniles, whereas 5.7S IgY were detected only in the sera of the juveniles. Both monoclonal antibodies specifically recognized particular immunoglobulins in turtle sera and did not crossreact with either human

sera or milk components. Moreover, these results encouraged us to test these turtle sera for the presence or absence of antibodies against ChHV5.

Production of F-US4-GST- and F-US8-GST fusion proteins

The construction of the bacmids for these baculoviruses has been described in part 1. After transfecting the bacmids into Sf9 cells, recombinant baculoviruses were generated and titrated as described in part 2. The products secreted to the supernatants of conventional as well as mimic Sf9 cells infected with these baculoviruses were harvested and analyzed by immunowesternblot, using the mcAb against the C-myc tag. Exemplary results are shown in Fig. 7. In lanes 1 and 2, a major band of F-US4-c-myc-GST migrated at around the 55 kDa marker band, which corresponded with the predicted molecular weight of 47.9 kDa. This result confirmed that the desired F-US4 fusion protein was secreted to the supernatant of the infected cells. However, faster migrating bands, which may represent degradation products, could be observed in between of the 25 kDa and the 35 kDa markers.

The results for the F-US8-c-myc-GST fusion protein (Fig. 7, lanes 3 and 4) were similar. A major band migrated in between of the 70 and the 100 kDa marker bands, which corresponded with the predicted molecular weight of 73.4 kDa F-US8-fusion protein. In addition, several faint bands running at around 70, 35 and 25 kDa may represent break-down products of the fusion protein. It was concluded, that the F-US8 fusion protein was secreted to the cell-free supernatant. Interestingly, there was no difference in mobility between the proteins expressed in conventional Sf9 cells as compared to the protein expressed in mimic Sf9 cells unlike the construct F-US4-V5-6his produced for the immunization experiments.

Titration of F-US4-GST- and F-US8-GST fusion protein antigens on casein-gluthation-coated ELISA plates

To test the gluthation-binding capacity of the GST-fusion proteins, an ELISA plate was coated with gluthation-conjugated casein as described in Materials and Methods. After washing and blocking, separate wells of the plate were incubated with either no antigen (solely dilution buffer) or with supernatant from mimic SF9 cells infected with either F-US4-c-myc-GST or F-US8-c-myc-GST or mock-infected. Starting from a dilution of 1:25, serial 2-fold dilutions were applied up to the final dilution of 1:51200. After incubating and washing, the binding of the fusion proteins was visualized by use of a mcAb against C-myc and a peroxidase-conjugated anti-mouse antibody. The results are shown in Fig. 8. The 1:25 diluted supernatant reached an OD450 of almost 3.0, while a serial dilution of the bound fusion protein was indeed observed, until it reached background level at a dilution of approximately 1:1600. At the low dilutions (1:25 and 1:50) some background reaction of the two negative controls was observed. However, at 1:100, the non-specific reaction had reached background level, while the two antigens of interest, F-US4 and F-US8 still caused a reaction of more than 1.0 OD 450nm. These observations confirmed that the two fusion proteins carried a functional GST-tag, which allowed to quantitatively bind the native forms of the proteins to the casein-glutathion-coated plates.

Analysis of sera from Australian turtles

Having established that the sera of hatchlings as well as of juvenile turtles from Australia contained antibodies that can be detected by mcAb CO2 against turtle 7S IgY, and that F-US4 and F-US8 fusion proteins could be bound quantitatively and in a native state to ELISA plates, it was of interest to test these available turtle sera for antibodies against the two ChHV5 antigens. For this purpose, ELISA plates were coated with casein glutathione, followed by adsorbing three different antigens and a negative control per serum, i.e. F-US4-c-myc-GST, F-US8-c-myc-GST, canine-papillomavirus-1-capsid-protein-GST (CPV1), and supernatant from mock-infected insect cells. A milk solution in the place of serum was used as a negative control. A mcAb against GST was used to measure binding of the GST-fusion proteins to the ELISA plates and demonstrate absence of GST in the case of the supernatant from the uninfected cells. The results are shown in Fig. 9, divided into 9A, showing the reactions of the hatchlings and 9B, showing the reactions of the juvenile turtles. Notably, the mcAb against GST caused an OD 450 nm of 2.0445 with the F-US4 fusion protein, 1.896 with F-US8, 1.5866 with CPV1, and 0.0925 with supernatant from uninfected cells. These data confirmed that for all three positive antigens ample GST-fusion protein had bound to the plates, whereas no GST was detected bound after incubating with the uninfected cell supernatant. The reactions of the turtle sera were indiscriminately low, almost exclusively below OD 0.1. The highest reaction (0.1905) was observed with CPV1 antigen. Statistically, there was no difference between the binding of any antiserum against any antigen. We concluded from this that none of the available sera contained any detectable 7S IgY against either F-US4 or F-US8.

Testing of selected Hawaiian turtle sera

Next, it was of interest to test sera from turtles with FP as well as appropriate controls for antibodies against F-US4 and F-US8 by using the same ELISA. For this purpose, a collection of 60 sera had been made available: 20 sera originated from Big Island (#1-20), where FP is rarely observed. 40 sera had been collected in Kaneohe Bay, where FP is observed frequently. 20 of those latter sera came from healthy turtles (#21-40) and 20 from turtles with FP (#41-60). The results, sorted according to location and serum number, are shown in Fig. 10. Apparently, the highest reactions were observed among the turtles with FP, whereas sera from turtles without FP showed clearly less reaction. A quantitative analysis of the data (Fig. 11) indicated that turtles with FP had significantly more antibodies against F-US4 and F-US8 than turtles without FP. This result was true for both 7S and 5.7S IgY.

As no gold standard for measuring antibodies against ChHV5 exists and since -- at least in the absence of FP -- the true individual status concerning infection with ChHV5 cannot be known, it was not possible to draw a reliable cut-off value for our test to discriminate infected from non-infected animals. However, it is remarkable that healthy animals from Kaneohe Bay showed barely a seroreaction, while many of the FP animals from the same location showed a very high seroreaction against both viral antigens.

Discussion

The Chelonid herpesvirus 5 (ChHV5) is considered the causative agent of fibropapilloma (FP) in marine turtles. In this project, we developed an ELISA to detect anti-ChHV5 antibodies in turtles against F-US4 and F-US8, respectively. F-US4 represents probably the major receptor binding protein, whereas F-US8, as a predicted homolog to herpesvirus gE, was suspected to be immunogenic but non-essential for ChHV5 replication. Furthermore, we developed another ELISA to detect two IgY variants in turtle sera, i.e. 7S IgY and 5.7S IgY. This was important in order to judge the immunocompetence of different turtle age groups. These newly created ELISAs were used to screen a selection of Australian hatchling and juvenile turtles for the presence or absence of 7S IgY and 5.7S IgY antibodies before testing them also for presence or absence of anti-F-US4 and anti-F-US8 antibodies. Moreover, the F-US4 and F-US8 ELISA was also applied on Hawaiian turtle sera, collected from animals with or without FP. Finally, soluble F-US4 and F-US8 antigens were produced and purified in order to vaccinate marine turtles for the production of reference sera.

The salient features of this study are as follows:

- Recombinant baculoviruses were constructed to express the exodomains of F-US4 and F-US8, respectively. The recombinant proteins, comprising a c-myc- and a GST-tag, were used to develop an ELISA, in which glutathione-conjugated casein was coated to accommodate the binding of equimolar amounts of GST-fusion proteins as ELISA antigen in a native state.
- Variants of the above proteins, comprising a C-terminal V5-tag for identification followed by a 6xhis-tag for purification, in the place of the c-myc- and GST-tags, were secreted to the supernatant of baculovirus-infected insect cultures and could be purified to a homogeneity of about 90%.
- Monoclonal antibodies were used to detect two particular IgY variants in turtle sera, i.e. the CO2 mcAb detected 7S IgY, whereas the D70 mcAb detected 5.7S IgY (Work et al., 2015). A pilot study using Australian turtle sera revealed that sera from young hatchlings (approximately 4 weeks old) contained low amounts of 7S IgY, whereas 5.7S IgY remained undetectable. In contrast, juvenile turtles (10 years or older) had ample amounts of both types of antibodies. Protein-G-binding antibodies were detected at low amounts and exclusively in the sera of the juvenile turtles. One individual, a few weeks older than the rest of the hatchlings, gave signals that were between the signals of the two groups (hatchlings and juveniles), showing, that both 7S and 5.7S IgY develop rapidly during the first few months of life. It was concluded, that immunocompetence was reached at the age of of about 12 weeks. This result was important for us to know, at what age to start with the immunization experiment.
- Using the Casein-glutathione-GST-ELISA, the same turtle sera were also tested for antibodies against either F-US4 or F-US8 but with negative results. However, when Hawaiian turtle sera were tested in the same ELISA, high seroreactors were detected, particularly among animals with a high load of FP-tumors. Interestingly, tumor-free animals carrying clearly detectable amounts of antibodies against F-US4 and F-US8 were hardly detected, not even among animals co-habitating in the same area as seropositive animals with tumors.

Normally, neutralization assays represent the Gold standard for serology against viruses (Holz et al., 2010). However, this is not possible with ChHV5 because this virus does not replicate in conventional cell cultures (Work et al., 2009; Work et al., 2017). Therefore, an ELISA may represent a good alternative. Yet, previous attempts to establish ELISA-based serologies for turtle herpesviruses yielded contradictory results (Coberley, 2001; Herbst, 2008). Herbst (2008) developed an ELISA to detect anti-ChHV5 antibodies in turtles by using a Baculovirus-expressed glycoprotein H (gH, F-UL22) as the coating antigen. Using captive reared green turtles (Chelonia mydas) with no history of virus exposure as "known negatives" and others with experimentally induced FP as "known positives," this assay had, apparently, a high specificity but a low sensitivity. Interestingly, seroconversion in experimentally with ChHV5-inoculated turtles was detected in only half of the animals of those bearing experimentally induced tumors. Indeed, antibodies were detected only in samples collected after the development of cutaneous fibropapillomas. However, when the same assay was applied to sera collected from wild green turtles in three Florida localities with different FP prevalences, including one site with no history of FP, approximately 80% of all sera from each single site gave a positive reaction against the Baculovirus-expressed glycoprotein H. As herpesviruses other than ChHV5 are widely prevalent among Florida green turtles (Herbst et al., 2008, Coberley et al., 2001), it remained unclear how these observations should be interpreted. The authors offered two explanations, i.e. either ChHV5 was highly prevalent among non-diseased turtles or a thus far undetermined herpesvirus caused a serious cross-reaction in this assay.

In our approach, we introduced four differences to the assay developed by Herbst and coworkers: (1) Instead of the glycoprotein gH antigen chosen by Herbst et al. (2008), we had selected two different viral glycoproteins, namely F-US4, which bears a certain similarity to gD of other alphaherpesviruses and F-US8, which resembles gE (Ackermann et al., 2012).

The choice of not using gH as an antigen was influenced by the knowledge that during viral replication the glycoprotein gH heterodimerizes with another glycoprotein gL, which leads to conformational changes in both of the interaction partners, which again is known to be associated with the formation of conformational epitopes for antibody binding (Hutchinson, 1992)).

Alternatively, the choice of the putative gE homolog F-US8 as ELISA antigen was influenced by the experience that gE of other alphaherpesviruses is known as a very strong antigen (van Oirschot, 1995; Weiss, 2015; Jacobs, 1994)). The marker vaccines against the Pseudorabies virus (PRV) in pigs and the Infectious Bovine Rhinotracheitis (IBR) virus in cattle both use gE-deletion viruses as vaccine strains because gE is highly immunogenic but non-essential for replication of these viruses in cell culture, while gE-deletion viruses cannot establish efficient circulation in nature (Jacobs, 1994)). However, gE deletion viruses were still able to confer a reliable protection against disease upon superinfection with a gE-positive wild type virus. Based on these properties it was possible in both cases to develop vaccines, which allowed the discrimination of vaccinated and naturally infected animals, i.e. so called DIVA-vaccines.

The choice of F-US4 as ELISA antigen was based on different aspects. Previously, our group had postulated that F-US4 may represent a homolog to gD, the receptor-binding protein of many alphaherpesviruses (Ackermann et al., 2012). Antibodies against the receptor-binding protein may be of great biological significance because they may neutralize viral infectivity. Accordingly, presence of such antibodies may correlate to immunological protection or else their absence may signify a particular vulnerability against the virus. Moreover, the nature of

viral receptors on host cells greatly influences the host's susceptibility to the corresponding virus and is a major determinant for viral pathogenesis. Accordingly, immunity against the viral receptor-binding protein may correlate with immunity against viral disease.

(2) Another difference was the binding of our antigens via their C-terminal GST-tag to the glutathione-casein coated plate, which enabled us to keep the antigens in native state (Sehr, 2001 & 2002). This is in contrast to conventional ELISAs, such as those used by Herbst (2008), which bind their antigen directly and under denaturing conditions to the plastic surface of the ELISA plate (Gibbs, J., ELISA Technical Bulletin, life sciences). Antigens in their native conformation may be more suitable to discriminate infections with closely related viruses, for example the GST-fusion protein approach is frequently used in the papillomavirus field, where the capsid protein L1 spontaneously assembles into virus-like particles, which in turn allow the discrimination of infections with particular viral serotypes (Lange et al., 2009; Sehr, 2002). As Herbst (2008) had postulated the potential interference of a thus far undetermined chelonid herpesvirus, it was of interest to use the present approach with the aim to potentially avoid cross-reactions.

(3) Our antigens were produced either in conventional sf9 insect cells or else in mimic sf9 cells to achieve a more complex, "humanized" glycosylation pattern than antigens produced in conventional insect cells (Thermo Fisher Scientific user guide). Indeed, antigens produced from mimic sf9 cells had a higher molecular weight than those produced from conventional sf9 cells (see Fig. 3), which may be important both for inducing and detecting a specific antibody response in turtles, since turtles, similar to mammalians, make use of a more complex glycosylation pathway than insects (Hirokazu Yagi, 2010).

(4) While Herbst et al (2008) tested only for 7S antibodies against ChHV5, we extended the range of antibodies to both 7S and 5.7S. As reported previously, a 5.7S antibody response develops much more slowly than a 7S antibody response and may be indicative for a chronic antigenic stimulation (Herbst and Klein, 1995; Work et al., 2000; Work et al., 2015). Before screening sera of turtles of different age groups, namely hatchlings, juveniles and adults, for the presence of anti-ChHV5 antibodies, we wanted to know, if both types of IgY are present in the sera of juveniles and especially hatchlings in the first place. So we developed an ELISA to detect these two particular antibody types, 7S and 5.7S IgY in the sera of these two age groups. We detected that hatchlings had low amounts of 7S IgY, while 5.7S IgY were below the limit of detection in the same sera. In contrast, both 7S and 5.7S IgY were readily detected in the sera of juvenile turtles (Fig. 6). In agreement with previous studies, we were able to confirm that some IgY in turtle sera can be detected by protein G (Work et al., 2015). However, the amounts of protein G-binding IgY were low in the juveniles and below the detection limit in the hatchling sera. Importantly, this series of experiments also confirmed previous reports that our anti-IgY monoclonal antibodies actually were specific for turtle IgY and did not cross-react with immunoglobulins of other animal species (Work et al. 2015).

One of the difficulties in our study was that we did not get permission for experimental inoculation of turtles with ChHV5 in order to obtain a collection of gold-standard sera, which would allow us to clearly discriminate seronegative from seropositive. However, we used a control antigen, comprising the tags (c-myc and GST), which was able to bind to casein-gluthatione-coated ELISA plates. The c-myc tag, present on each variety of ELISA antigen, was further used to coat comparable amounts of antigens to the plate. Since each gluthatione molecule can bind one GST-counterpart and each fusion protein comprised one singly c-myc

epitope, dilutions of each antigen were incubated on casein-gluthatione-coated ELISA plates before the amount of bound antigen was determined using an mcAb against c-myc. Afterwards, each antigen was diluted in a manner to give an equal ELISA reaction upon testing with the c-myc mcAb, thus, providing equimolar coating of each antigen. On one hand, a serum was considered positive, if its reaction was obviously stronger with the ChHV5-fusion protein than with the fusion protein without ChHV5-part. On the other hand, a serum was considered negative, if the reactions against the ChHV5-less antigen did not greatly differ from its ChHV5-containing counterpart. Thus, while a clear cut-off value to discriminate between positive and negative could not be established, it was possible to discriminate between sera with strong reactions and sera with weak or no reactions against the ChHV5-containing antigen.

Based on these conditions, the results of our study were only in part consistent with the previous findings by Herbst and others (2008). Consistent with that study, we did not detect ChHV5 antibodies in captive reared turtles without history of FP. Moreover, clearly seropositive animals were found almost exclusively among animals with FP, which is coherent with Herbst's finding that experimentally infected animals developed antibodies only after the development of tumors. Yet in stark contrast to that previous study, where more than 80% anti-gH-antibody-positive animals existed among populations without history of FP, our assay did not detect many FP-negative but ChHV5-seropositive animals in the wild. Interestingly, this was true for sera taken either from Hawaiian or from Australian turtles. Notably, most sera that were considered "positive" reacted equally strong against the F-US4- and the F-US8-fusion protein. Moreover, 7S and 5.7S reactions were equally often detected but, in general, the 5.7S IgY gave stronger reactions than the 7S antibodies. Antigens produced on conventional Sf9 cells created some more background reaction than antigens produced on Sf9 mimic cells but neither cell type did give rise to conflicting results.

The general scarcity of clearly seropositive individuals among Hawaiian and Australian turtles came as a surprise. Herpesviruses that freely circulate among humans and other animals provide usually a serological prevalence of higher than 50%, which includes a majority of individuals without disease signs (Wald, Corey, 2007). For example, a recent study on the prevalence of antibodies against human herpesviruses among Swiss people without any disease signs revealed that over 50% had antibodies against HSV-1 and HCMV, whereas the seroprevalence of healthy individuals with antibodies against VZV, EBV and HHV6 was higher than 80% (Barandun, 2014). Only HSV-2 and HHV8 showed seroprevalences of 20% or less, which was to be expected since, due to their biological properties, both of these viruses do not circulate freely among the Swiss population (Bünzli, 2004; Regamey, 1998).

Together, these observations and considerations may contribute to an already controversial discussion about the prevalence, disease-causing role, and natural reservoir of ChHV5. According to one scenario, which is at least in part supported by Herbst's (2008) serology data, ChHV5 is widely distributed and causes FP only in a few of many infected animals. Consistent with this, a variety of groups has reported frequent detection of ChHV5 DNA on the skin of seemingly healthy turtles. Interestingly, this finding is confirmed predominantly among groups that work on Atlantic and Caribbean turtles (Page-Karjian, 2012; Alfaro-Núñez, 2014). In contrast, groups working on Pacific turtles, with most studies around the Hawaiian Islands, were consistently unable to detect ChHV5 DNA from non-tumorous tissues (Work, 2015). Fascinatingly, this discrepancy is now mirrored in the serological studies, which suggest high prevalence of the virus in Florida and very low prevalence in Hawaii and Australia. As explanation for this seeming contradiction one may argue that over decades, the treatment of

animals with FP has been vastly different for either the Atlantic or the Pacific turtle population. While Pacific turtles with FP were left without treatment or were euthanized, surgical removal of FP tumors has been quite common to treat Atlantic turtles (Brunner, 2014). Ultimately, this may have led to the development of a ChHV5 variant virus, which is high contagious and, thus, highly prevalent but probably less virulent with regard to causing FP. Such a variant, which would be restricted to the Atlantic areas might explain the different prevalence findings in Atlantic and Pacific regions.

However, these considerations lead also to an alternative scenario, according to which a virus with high similarity to ChHV5 may exist, though circulating only among Atlantic turtles, which could be responsible for the serological cross-reactions observed among Florida turtles and which could explain the detection of viral DNA on the skin of healthy animals in these regions. Indeed, herpesviruses other than ChHV5 have frequently been detected among Atlantic turtles but do apparently not circulate among Hawaiian or Australian turtles (Stacy, 2008). If this second scenario were true, the question would arise as to whether or not ChHV5 is actually a turtle virus. It is difficult to perceive that ChHV5 has been able to mount an FP-pandemic with only so few animals excreting and transmitting virus (Work et al., 2015). An obvious explanation for this would be that animals other than turtles may represent the true virus reservoir. Indeed, thus far only two reports exist, which conceivably describe the replication of ChHV5 in turtle tissue (Work et al., 2015; Work et al., 2017).

Consistent with the above considerations, we did not detect anti-ChHV5 antibodies among a selection of Australian turtles, neither in hatchlings nor in juvenile individuals from different locations (Fig. 9).

For the immunization experiments in order to raise reference sera for our serological assay, it was planned to immunize turtles with either purified F-US4 protein or purified F-US8 protein. Originally it was planned to immunize juvenile turtles for the purpose of collecting reference sera. Unfortunately, the permission by the Australian authorities requested euthanasia of all vaccinated animals at the end of the experiments. Because only a small percentage of turtle hatchlings reaches the age of those juveniles (http://oceanservice.noaa.gov/), and because of the high value of these animals for future breeding, it was decided to immunize turtle hatchlings in the place of the juveniles, because their replacement is easier. Regarding immunizing hatchlings there are three issues that have to be considered. 1. To date, to our knowledge, there are no reports about vaccinating turtle hatchlings, only reports about successful immunizations of juvenile turtles are available (Benedict & Pollard, 1972; Work et al., 2000), 2. newborn animals are usually not fully immunocompetent (Hodgins, 2012). In reptiles the development of the immune system is even slower. It was detected, that the snapping turtle's complete immunological competence occurred only a few month after hatching (El Deeb, 1990). In previous studies about spirorchiid infection in green turtles, it was found, that hatchlings had significantly lower titers of 7S IgY against some spirorchiid antigens compared to juvenile turtles or adults (Work et al., 2005). Our investigations about the presence of immunoglobulins in turtle hatchlings confirmed that hatchlings may have IgY of the type 7S, though to a much lesser quantity than juvenile turtles. Work et al. suggested, that the low antibody titers in hatchlings against the spirorchiid antigens might represent maternal antibodies transferred to the offspring, because hatchlings are very unlikely exposed to trematode *cercariae* before leaving the nesting beach (Work et al., 2005). And this leads to the third issue regarding immunizing turtle hatchlings: 3. maternal antibodies, transferred from the mother to the offspring might interfere with the immunization process (Hodgins, 2012). But if the mother was

not ChHV5-seropositive, the hatchlings are not likely carriers of anti-ChHV5 antibodies. Specific antibodies against our two viral antigens F-US4 and F-US8 were not detected in our experiment. Therefore interference of maternal antibodies with the vaccination process had not to be expected in our case. Still, due to the lower immunocompetence at the time of the first immunization, there is a risk, that hatchlings develop an immunetolerance against those two particular antigens (Piccand, 2016; Sanchez-Schmitz, 2011). In our ELISA testing for the presence of IgY in turtle sera, the serum of one single, several months old turtle had also been analyzed but this results were not included in the Figure because of its higher age compared to the other hatchlings (around 4 weeks old). This turtle did not match into any of the two groups. Interestingly, signals detected were actually between the signals of the two groups, suggesting that both 7S and 5.7S IgY develop rapidly during the first few months of life. The higher antibody level compared to the younger hatchlings found in this specific turtle are clearly the result of own antibody production and shows the stage, where immunocompetence was reached. Therefore we found the right age of turtle hatchlings to start with the immunization process.

The proteins designed for that purpose were expressed in the cell culture supernatant, from which they could be purified on magnetic nickel beads under native conditions to a homogeneity of about 90%. With this purity, the vaccine was ready for the immunization experiments on turtles.

Conclusions:

We were able to develop an ELISA, in which glutathione-conjugated casein was coated to accommodate the binding of equimolar amounts of GST-fusion proteins as ELISA antigen in a native state. Results were reproducible and equally strong against the F-US4- and the F-US8-fusion protein. With our assay, clearly seropositive animals were found almost exclusively among animals expressing FP tumors, whereas FP-negative but clearly ChHV5-seropositive animals in the wild were barely detected. This was the case for sera taken from Hawaiian and from Australian turtles. The scarcity of clearly seropositive individuals among Hawaiian and Australian turtles was surprising and led to the conclusion that ChHV5 was either not circulating openly in the turtle population or antibody titers of seropositive but tumor-free animals were very low. Our immunization experiments, which are in progress, will eventually lead to the production of reference sera may help to draw a cut-off value to distinguish between seropositive and seronegative animals.

The ELISA results detecting turtle antibodies against two viral glycoproteins F-US4 and F-US8 of ChHV5 allowed further discussion about the prevalence and the natural reservoir of ChHV5. Different scenarios explaining the different prevalence findings in Atlantic and Pacific regions are possible.

The different treatment strategies of FP animals from either Atlantic or Pacific regions may have led to the development of a ChHV5 variant virus, which is very contagious and therefore highly prevalent among Atlantic turtle populations. Another possibility to explain the different prevalence findings in Atlantic and Pacific regions is the existence of a virus other than ChHV5 with high similarity to ChHV5 circulating among Atlantic turtle populations, leading to serological cross-reactions. These considerations led to the suggestion, that animals other than turtles may represent the true virus reservoir for ChHV5. In future experiments it would be interesting, to use our assay on sera of Atlantic turtle populations to see, if high prevalence

could be confirmed as previously tested (Herbst et al., 2008; Stacy, 2008). Moreover, other viral antigens could be expressed and used with the same ELISA protocol, in order to find a tool to distinguish FP-negative but ChHV5-seropositive animals from FP-negative animals that had never encountered ChHV5.

Additionally it would be interesting to test, if antibodies against F-US4 upon immunization are protective against development of the disease. This would be a first step towards control of the disease by using a vaccine containing F-US4 as an antigen.

Our finding that hatchlings have originally very low IgY concentrations in their sera and that these increase only several months after hatching, indicates that very young turtles are probably not fully immunocompetent and that too early vaccination may lead rather to tolerance than to immunity. Therefore, we suggest to vaccinate hatchlings in the future not before 6 months of age.

Figures

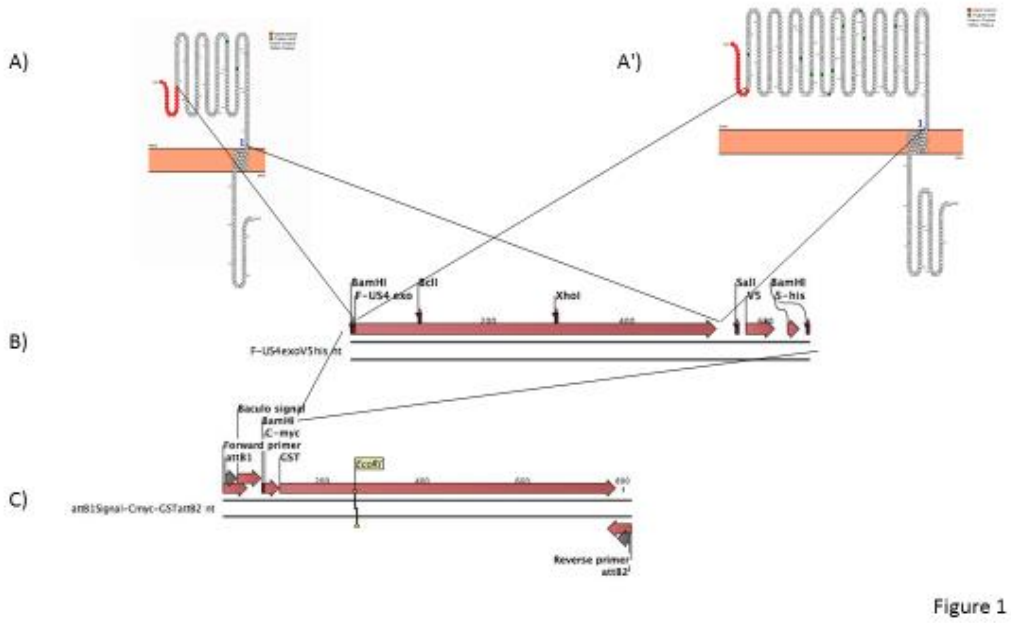


Figure 1

Fig. 1. Strategy of vector construction.

(A) Schematic representation of the F-US4 glycoprotein, inserted into a membrane (orange bar). The cytoplasmic tail (below the membrane), the transmembrane region, and the signal sequence (in red) were excluded from the construct, which thus comprised exclusively the domain protruding from the outer side of the membrane (exo-domain). (Pictures exported from PROTTER, Protter: interactive protein feature visualization and integration with experimental proteomic data. Omasits U, Ahrens CH, Müller S, Wollscheid B. Bioinformatics. 2014 Mar 15;30(6):884-6. doi: 10.1093/bioinformatics/btt607.)

(B) The amino acid sequence of the F-US4 exodomain (and F-US8; not shown) was reverse transcribed in silico, optimized for expression in insect cells, and synthesized in frame with two C-terminal epitope tags, namely V5 and 6-his. The entire construct was flanked by BamHI sites to fit the cassette into the construct in (C). The V5-6xhis tail was followed by a STOP codon. A variant of this construct was synthesized, in which the V5-6xhis tags were deleted and which came without STOP codon to provide a fusion protein with the C-terminal C-myc-GST tail. Similar variants were constructed for the F-US8 exodomain (A').

(C) The Signal-C-my-GST construct was synthesized to be flanked by attB1 and attB2 sites, respectively, for transposition into the Gateway entry vector pDONR221. It also contained one single BamHI site in between of the synthetic baculovirus signal sequence and the C-myc tag, which served to accommodate insertion of various BamHI fragments, whose amino acid templates are depicted in A and A'. (Pictures B and C exported from CLC Main Workbench 7)

Primer list				
attB1sigBac	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAGGGTGTTGGTTCTGTT 3'			
GST_attB2	5' GGGGACCACTTTGTACAAGAAAGCTGGGTTTATCTTGGGACGAGGTCAC 3'			
Primer M13	5' TGTAAAACGACGGCCAG 3'			
Primer M13r	5' CAGGAAACAGCTATGAC 3'			
PH_profor	5' AAATGATAACCATCTCGC 3'			
SV40pA-rev	5' GAAATTTGTGATGCTATTGC 3'			
pUCM13F	5' CCCAGTCACGACGTTGTAAAACG 3'			
pUCM13R	5' AGCGGATAACAATTTCACACAGG 3'			
att sites				
attB1 site	5' – ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-T – 3'			
attB2 site	5' – AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GT – 3'			
Viral titers in Pfu/ml				
	F-US4-V5-6his	F-US8-V5-6his	F-US4-c-myc-GST	F-US8-c-myc-GST
P0 stock	10^5.5 Pfu/ml	10^5 Pfu/ml	10^5.25 Pfu/ml	10^4.75 Pfu/ml
P1 stock	10^6.5 Pfu/ml	10^6 Pfu/ml	10^6 Pfu/ml	10^5.75 Pfu/ml
P2 stock	10^8 Pfu/ml	10^7.25 Pfu/ml	10^7.5 Pfu/ml	10^7 Pfu/ml

Table 1

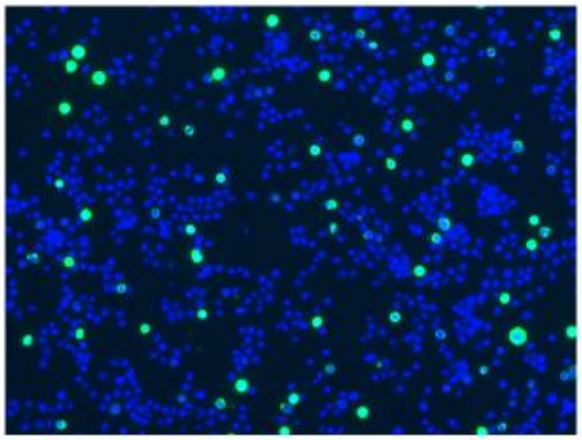


Figure 2

Fig. 2. Identification of recombinant baculoviruses. Sf9 cells were transfected with 1 µg bacmid DNA and propagated for 96 hrs. One replicate sample of the transfected cells was fixed with 4% PFA for 15 minutes and permeabilised in PBS, 0.1% Triton-X100 before anti-V5 monoclonal antibody was added, followed by alexa fluor 488 goat-anti-mouse (green fluorescence). The nuclei of the cells were counter stained with DAPI (blue fluorescence) prior to analysis under the fluorescence microscope. (Magnification: 20x; bar)

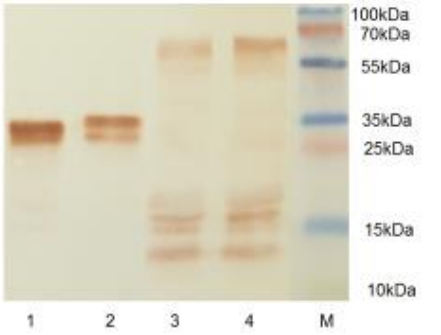


Figure 3

Fig. 3. Detection of V5-fusion proteins by Western immunoblot. Supernatants of baculovirus (lanes 1 and 2: F-US4-V5-6his; lanes 3 and 4: F-US8-V5-6his) inoculated cell cultures (Sf9 cells: lanes 1 and 3; mimic Sf9 cells: lanes 2 and 4) were separated by PAGE, transferred to PVDF membranes and immunostained with anti-V5 mcAb. Lane M: protein ladder with molecular weights indicated on the right.

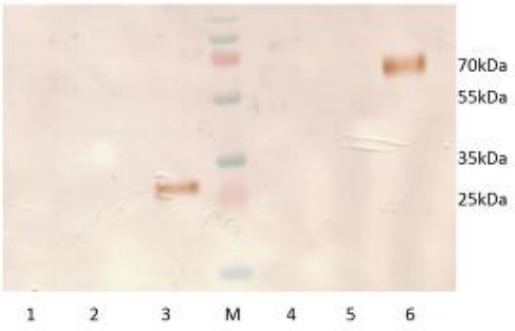


Figure 4

Fig. 4. Ni-purification of fusion proteins. Samples of the flow through fractions (lanes 1, 4), final washes (lanes 2,4), and eluates (lanes 3, 6) of magnetic nickel bead purification were separated by PAGE, transferred to PVDF membranes and immunostained with anti-V5 mcAb. Lane M: protein ladder with molecular weights indicated on the right.

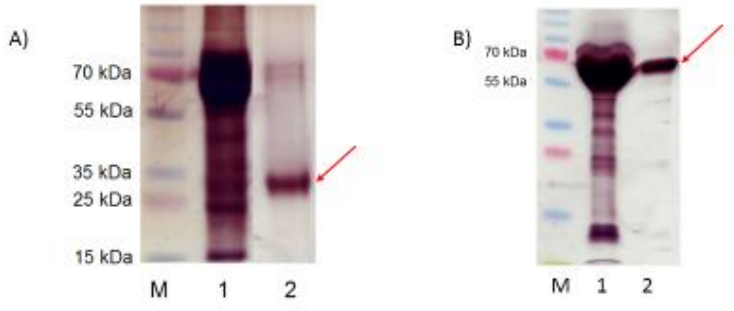
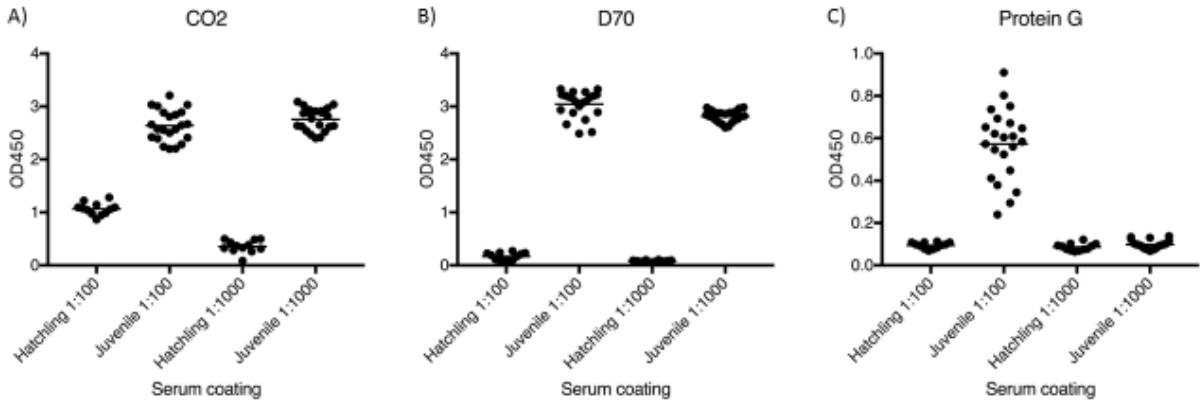


Figure 5

Fig. 5. Fusion proteins before and after Ni-purification. Samples of the starting materials (lanes 1) and of the final eluates (lanes 2) of the F-US4-V5-6his (A) and F-US8-V5-6his (B) fusion proteins were separated by PAGE and subjected to silver staining. M indicates the molecular weight marker. Relevant MWs are indicated on the left of the pictures. The arrows point to the purified products.



	human 1:100			human 1:1000	
co2	0.068	0.0798	co2	0.0619	0.0611
d70	0.0599	0.1541	d70	0.0512	0.0902
pg	1.70809996	0.1145	pg	0.97049999	0.0708

Figure 6

Fig. 6. Age dependent antibodies in turtle sera.

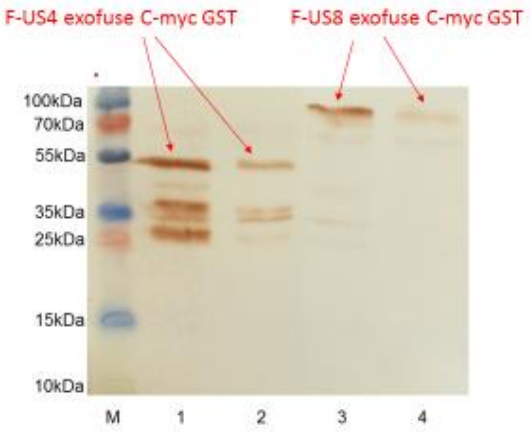


Figure 7

Fig. 7. GST-fusion proteins as ELISA antigens. Detection by Western immunoblot. Supernatants of baculovirus (lanes 1 and 2: F-US4-C-myc-GST; lanes 3 and 4: F-US8-C-myc-GST) inoculated cell cultures (Sf9 cells: lanes 1 and 3; mimic Sf9 cells: lanes 2 and 4) were separated by PAGE, transferred to PVDF membranes and immunostained with anti-C-myc mcAb. Lane M: protein ladder with molecular weights indicated on the left.

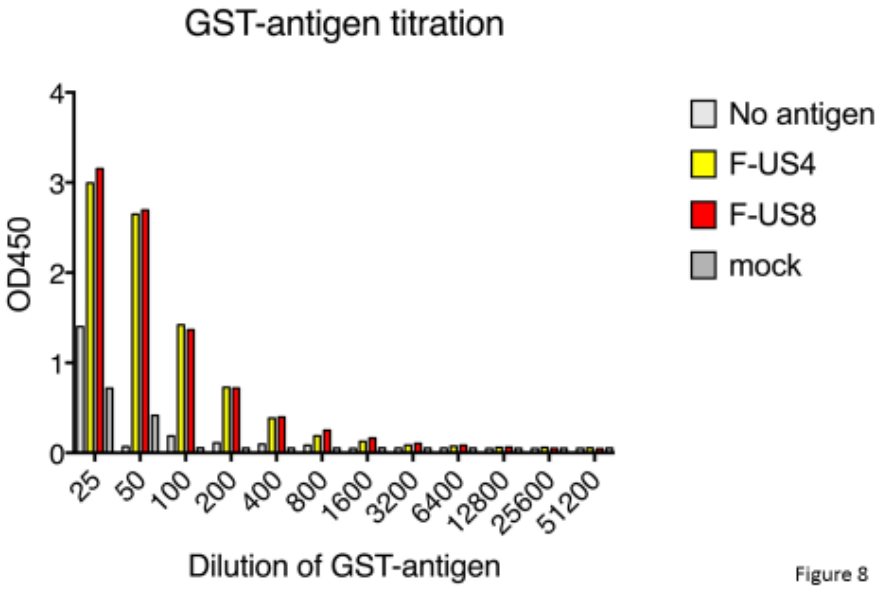


Figure 8

Fig. 8. Dilution of GST-antigens on casein coated plates. Detected by anti-C-myc-mcAb reaction

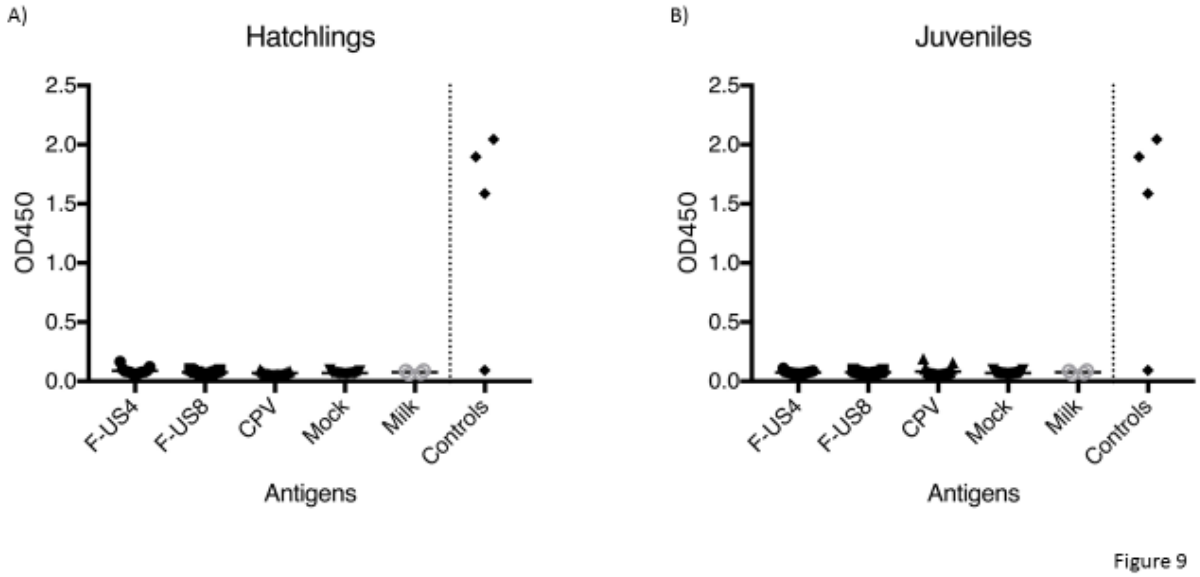


Fig. 9. GST-ELISA (mcAb CO2 against 7S IgY).

anti-GST antibody reacted as follows (OD450) with the four antigens:

F-US4: 2.0445

CPV: 1.5866

uninfected supernatant: 0.0925

F-US8: 1.896

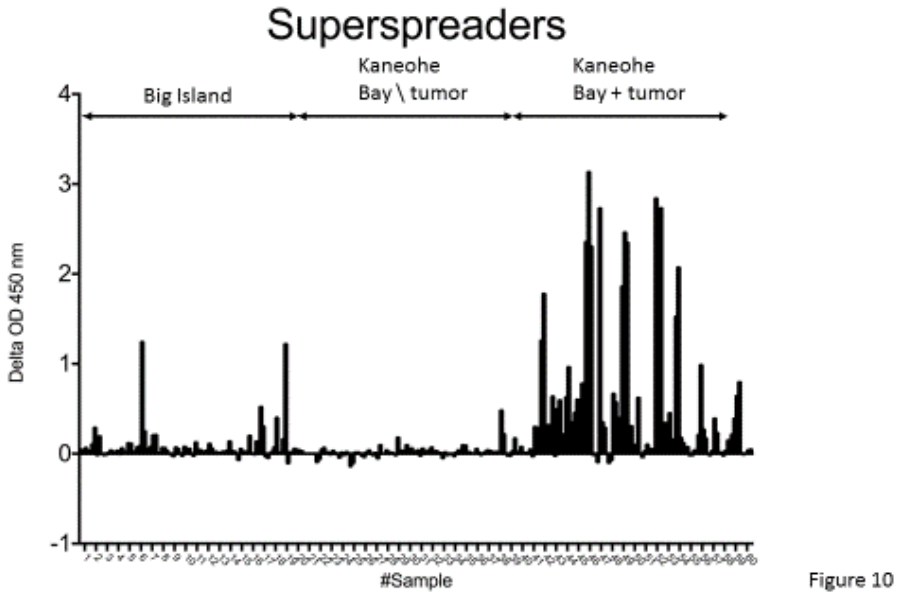


Fig. 10. F-US4-GST-ELISA using sera from turtles with or without tumor.

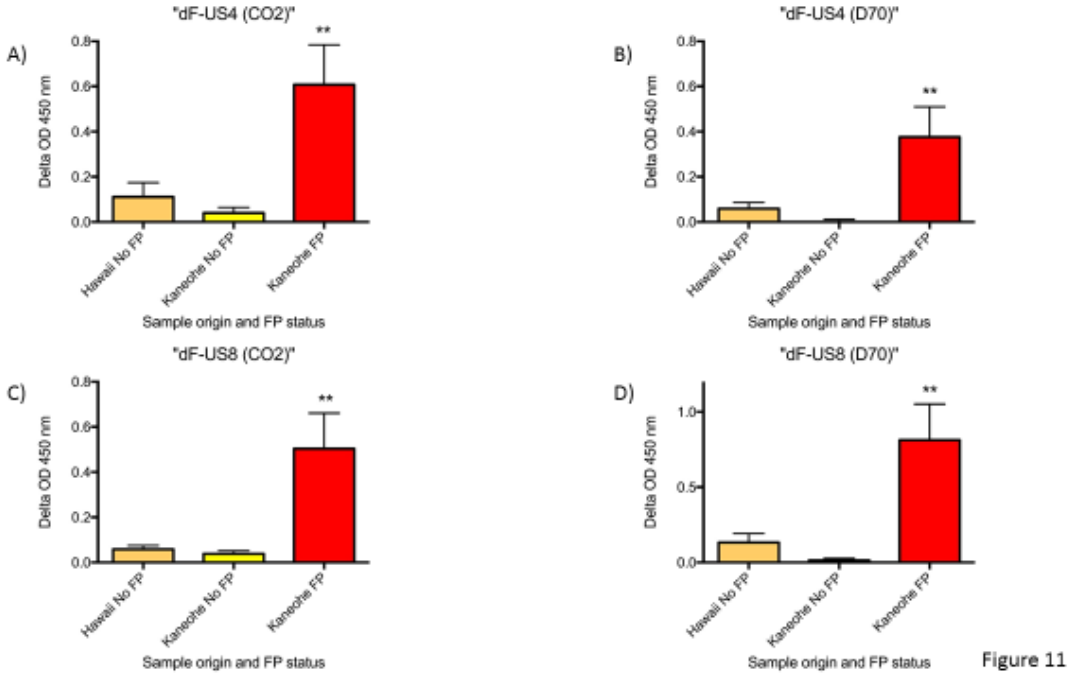


Fig. 11. Association of fibropapillomatosis with antibodies against ChHV5.

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